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Isolation and characterization of Ni mobilizing PGPB from serpentine soils and their potential in promoting plant growth and Ni accumulation by *Brassica* spp.

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ABSTRACT

The study was undertaken to assess the effects of Ni mobilizing bacteria on the plant growth and the uptake of Ni by *Brassica juncea* and *Brassica oxyrrhina*. Among a collection of Ni resistant bacterial strains isolated from the non-rhizosphere and rhizosphere soils of *Alyssum serpyllifolium* and *Astragalus incanus* at a serpentine site in Bragança, north-east of Portugal, nine strains were selected based on their ability to solubilize Ni in soil. Further assessment on plant growth-promoting parameters revealed the intrinsic ability of the Ni mobilizing strains to produce indole-3-acetic acid (IAA), siderophores, utilize 1-amino-cyclopropane-1-carboxylic acid (ACC) as the sole N source and solubilize insoluble phosphate. All of the strains tested positive for IAA production and phosphate solubilization. In addition, all the strains, except SRS5 exhibited significant levels of siderophore production. Besides, five isolates showed positive for ACC deaminase activity. In pot experiments, inoculation of plants with Ni mobilizing strains increased the biomass of both *B. juncea* and *B. oxyrrhina*. Among the strains, *Pseudomonas* sp. SRI2, *Psychrobacter* sp. SRS8 and *Bacillus* sp. SN9 showed maximum increase in the biomass of the test plants. In addition, the strain SN9 significantly increased the Ni concentration in the root and shoot tissues of *B. juncea* and *B. oxyrrhina*. Further, a significantly positive correlation was observed between the bacterial Ni mobilization in soil and the total Ni uptake in both plant species. The findings, therefore, revealed that inoculation of Ni mobilizing plant growth-promoting bacterial strain SN9 increases the efficiency of phytoextraction directly by enhancing Ni accumulation in plant tissues and indirectly by promoting the shoot and root biomass of *B. juncea* and *B. oxyrrhina*.

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1. Introduction

The contamination of soils with metals especially Ni is a major environmental problem throughout the world. Soils polluted with Ni may threaten ecosystems and human health. The remediation of soils contaminated with metals is a challenging task because metals cannot be degraded and the danger they pose is aggravated by their almost indefinite persistence in the environment. Conventional cleanup technologies are generally too costly to be used to restore contaminated sites, and are often harmful to the normal properties of the soil (i.e., texture and organic matter) (Holden, 1989). The emerging phytoremediation techniques, with their lower cost and environmental friendly nature, have received increasing attention in the last decades (Kumar et al., 1995).

The success of the phytoremediation process, whereby metals are effectively removed from soil, is dependent on an adequate yield of plants and on the efficient transfer of metals from the roots of the plants into their shoots. Most hyperaccumulators, such as *Thlaspi*, *Urtica*, *Chenopodium*, *Polygonum sachalase* and *Alyssum* are characterized by slow growth and low-biomass production, which make these plants impractical for use in phytoextraction in the field (Puschenreiter et al., 2001). Hence, recent research projects on phytoextraction have focused on high-biomass crop species, such as maize, peas, oats and Indian mustard, and on relevant plant husbandry and soil management practices to enhance the metal uptake of these high-biomass species (Chen et al., 2004). Although several conditions must be met in order for phytoremediation to be effective, the bioavailability of metals to plant roots is considered to be a critical requirement for plant uptake to occur (Kayser et al., 2000). Soil factors such as pH, cation exchange capacity, or organic matter content play an important role in successful soil remediation processes. In recent years, several synthetic chelators such as EDTA have been suggested to enhance phytoextraction efficiency in metal-contaminated soils (Puschenreiter et al., 2001). However, chelator application in chemically assisted phytoextraction may also have potentially environmental risks. For instance, some chelators themselves are usually phytotoxic, and increasing metal solubility by them may be also phytotoxic to non-hyperaccumulator plants, thus plant growth may be inhibited, and the chance of success with chemically assisted phytoextraction may be lowered (McGrath and Zhao,

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2003). Therefore, the potential use of alternative methods that exploit rhizosphere microbes to enhance phytoextraction potential of hyperaccumulating plants has been investigated (Abou-Shanab et al., 2003; Pal et al., 2005). The metal resistant microbes in rhizosphere can affect trace metal mobility and availability to the plants through release of chelating agents, acidification, phosphate solubilization and redox changes (Abou-Shanab et al., 2003; Idris et al., 2004). For example, chemolithotrophic bacteria have been shown to enhance environmental mobility of metal contaminants via soil acidification, or in contrast, to decrease their solubility due to precipitation as sulfides. In addition, the naturally occurring bacteria in soils, contaminated with nickel have acquired resistance against nickel and other metals (Pal et al., 2005). Furthermore, these metal resistant bacteria have exceptional ability to promote the growth of the host plant by various mechanisms, namely fixation of atmospheric nitrogen, production of siderophores, solubilization of phosphate, or production of plant growth regulators (hormones) (Glick et al., 1998; Abou-Shanab et al., 2005). Therefore, such organisms endowed with Ni mobilizing ability and plant growth-promoting activities are of practical importance for efficient phytoremediation of metal-contaminated environment.

The objectives of this study were to isolate and characterize Ni resistant bacteria from Ni rich serpentine soils, and to select Ni mobilizing plant growth-promoting bacterial (PGPB) strains which might be useful to increase the plant Ni uptake and biomass production under unfavourable environmental conditions for improving the efficiency of phytoremediation of Ni-polluted soils.

2. Materials and methods

2.1. Experimental plant

Two *Brassica* sp., *B. juncea* and *B. oxyrrhina* were selected for this study based on their demonstrated ability to accumulate substantial amounts of metals in shoots (Kumar et al., 1995) and to produce substantial biomass in a very short time.

2.2. Isolation of Ni mobilizing bacteria

The bacterial strains were isolated from the non-rhizosphere and rhizosphere soils of *Alyssum serpyllifolium* and *Astragalus incanus* in serpentine soil at Bragança, north-east of Portugal, previously described by Freitas et al. (2004). Briefly, plants were harvested and their roots were shaken to remove the loosely attached soil. Soil adhering to the root was considered as the rhizosphere soil. The soil sample collected from unplanted serpentine area was considered as the non-rhizosphere soil. About 1 g of soil samples were serially diluted using 25 mM phosphate buffer and spread over on Luria-Bartani medium (LB) amended with 50 mg of Ni L^{-1} ($NiCl_2$). The plates were incubated at 37 °C for 48 h. From the Ni resistant colonies, different strains were picked and purified on the LB medium containing 50 mg L^{-1} of Ni according to the procedure of Rajkumar and Freitas (2008).

In order to isolate the Ni mobilizing bacteria, the Ni resistant strains were tested for the ability to increase the water soluble Ni concentrations in artificially Ni contaminated soils. The soil was collected from the Botanical garden, Department of Botany, University of Coimbra, Coimbra, Portugal. The soil was sieved (2 mm) and sterilized by steaming (100 °C for 1 h on three consecutive days). After sterilization the soil was amended with aqueous solution of $NiCl_2$ to achieve the final nickel concentrations of 450 mg Ni kg^{-1} and left in a greenhouse for a 2-week period (for metal stabilization). The Ni resistant strains were grown in LB broth and placed on a shaker at 200 rpm and 27 °C. After 24 h, optical density (600 nm) was measured and adjusted to 1.5; the cultures were centrifuged at 6000 rpm for 10 min, washed in phosphate buffer (pH 7.0) twice, resuspended, washed in sterile water twice, resuspended. Small aliquots of washed bacterial culture (up to 1 mL) were added to the 1 g of soil in the centrifuge tubes. Sterile water was added to soil as an axenic control. All tubes were placed on an orbital shaker at 200 rpm at 27 °C. After 5 d, 10 mL of sterile water were added to each tube to extract the soil water soluble Ni. The soil suspensions were centrifuged at 7000 rpm for 10 min and filtered. The concentrations of Ni in the filtrate were determined by atomic absorption spectrophotometer.

2.3. Genetic characterization of Ni mobilizing bacterial stains

The bacterial strains were grown in LB broth in presence of 1 mM Ni at 30 °C. Cells were harvested after 20 h and processed immediately for DNA isolation using standard procedure (Sambrook et al., 1989). Amplification of 16S rRNA gene sequence was performed by polymerase chain reaction (PCR) with the conserved eubacterial primers pA (5′-AGAGTTTGATCCTGGCTCAG; *Escherichia coli* bases 8–27) and pC5B (5′-TACCTTGTTACGACTT; *E. coli* bases 1507–1492) (Dunbar et al., 1999). Reaction conditions were as described by Branco et al. (2005). Each amplification mixture (5 μL) was analyzed by agarose gel (1.5% w/v) electrophoresis in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) containing 1 μg mL^{-1} (w/v) ethidium bromide. For further sequencing reaction, the amplified DNA was purified from salts and primers using the PCR purification kit (Roche Diagnostics) according to the manufacturer's instructions. Automated sequencing of the purified PCR products was performed using the dRodamina terminator cycle sequencing kit and the ABI 310 DNA Sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Partial 16S rDNA sequences obtained were matched against nucleotide sequences present in GenBank using the BLASTn program (Altschul et al., 1997).

2.4. Plant growth-promoting features of Ni mobilizing bacteria

The 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity of cell-free extracts was determined by estimating the amount of α-ketobutyrate (α-KB) generated by the enzymatic hydrolysis of ACC according to the procedure of Honma and Shimomura (1978).

Indole-3-acetic acid (IAA) production by Ni mobilizing bacteria was determined according to the method of Bric et al. (1991). Cultures of the isolates were raised in LB broth amended with 500 μg mL^{-1} of tryptophan at 27 °C for 120 h at 200 rpm. Cells were removed by centrifugation at 6000 rpm and the supernatant was assayed for IAA production.

Ni mobilizing bacteria were screened for the production of siderophores by the method of Schwyn and Neilands (1987) using chrome azurol S (CAS) agar. Briefly, the assay was performed by spotting 50 μL of each exponential bacterial culture previously grown under iron-restricted conditions in casamino acids (CAA) medium onto CAS agar. The siderophore levels produced by the isolates were recorded as the diameter of the orange halo produced by the colony. The presence of catechol and hydroxamate siderophores in culture supernatants obtained from bacteria grown either in iron-restricted conditions in CAA medium was quantitatively determined by the colorimetric assay of Arnow (1937), using 2,3-dihydroxybenzoic acid and the Atkin et al. (1970) method, using desferrioxamine mesylate as standards, respectively.

The phosphate solubilizing activity of the isolates was analyzed in modified Pikovskayas medium (Sundara-Rao and Sinha, 1963) amended with 0.5% of tricalcium phosphate. The isolates were grown at 27 °C for 120 h at 200 rpm. The solubilized phosphate

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in the culture supernatant was quantified as detailed by Fiske and Subbarow (1925).

2.5. Influence of Ni mobilizing PGPB on plant growth and Ni uptake

For pot experiments the soil collected from botanical garden was amended with aqueous solution of $NiCl_2$ to achieve the final Ni concentrations of 450 mg kg^{-1} as detailed in the earlier section. Before inoculation, the mutant of Ni mobilizing PGPB marked with antibiotic resistance were obtained after plating of the parental strain onto LB agar amended with ampicillin (75 mg L^{-1}). The seeds of *B. juncea* and *B. oxyrrhina* were inoculated by soaking in a bacterial suspension after adjusting $OD_{600\,nm}$ to 1 for 2 h. Seeds soaked in sterile water were used as control. The inoculated and non-inoculated seeds were planted in plastic pot (top diameter 120 mm, bottom 100 mm and height 90 mm) containing 300 g of soil. The plants were grown in a glasshouse at 25 °C and a 16/8 d/night regime. After 45 d the plants were carefully removed from the pots and the root surface was cleaned several times with distilled water. Growth parameters such as fresh weight and dry weight of the plants were measured. The accumulation of Ni in root and shoot system was also quantified following the method of Freitas et al. (2004). In addition, the population dynamics of introduced bacteria was studied using the intrinsic antibiotic marker. The adhering soil was removed from plant roots. For determination of rhizosphere soil colonization, 0.5 g soil removed from the roots was shaken with 10 mL sterile water for 30 min. The resulting suspensions were evaluated for colony forming units (cfu) according to the dilution-plate method on LB agar with addition of 75 mg L^{-1} ampicillin. The plates were incubated for 4 d at 28 °C. By adding ampicillin, the native bacterial flora was mostly excluded from the plates.

2.6. Statistical analysis

Analysis of variance followed by post-hoc Fisher LSD test ($p < 0.05$) were used to compare treatment means. Simple regression analysis was used to compare relationships between bacterial Ni mobilization in soil and Ni uptake in two plant species inoculated with bacterial isolates. All the statistical analyses were carried out using SPSS 10.0.

3. Results and discussion

3.1. Isolation and characterization of Ni mobilizing bacteria

Serpentine or ultramafic soils are produced by weathering and pedogenesis of ultramafic rocks that are characterized by high levels of Ni, Cr and sometimes Co, but contain low levels of essential nutrients such as N, P, K and Ca (Brooks, 1987). Numbers of plant species such as *A. serpyllifolium*, *Bromus hordeaceus*, *Linaria spartea* etc. endemic to serpentine soils are capable of accumulating exceptionally high concentrations of Ni (Freitas et al., 2004). However, the function of hyperaccumulation not only depends on the plant, but also on the interaction of the plant roots with rhizosphere microbes and the concentrations of bioavailable metals in soil. Currently, microorganisms present in serpentine soil and their interaction with serpentinophytes have attracted the attention of several investigators due to biotechnological applications for microbial-assisted phytoremediation (Abou-Shanab et al., 2005; Pal et al., 2005). Bacterial communities in serpentine soil were reported to tolerate spiking of metals, such as nickel, lead, copper and zinc (Abou-Shanab et al., 2005). Also, evidence was presented that the rhizosphere of hyperaccumulating plants, such as *Thlaspi goesingense* and *A. murale* has an increased proportion of metal resistant bacteria (Abou-Shanab et al., 2003; Idris et al., 2004). Furthermore, the serpentine bacteria have been shown to possess several traits that can alter heavy metal mobility and availability to the plants (Rajkumar and Freitas, 2008). Hence, in this investigation we isolated Ni mobilizing bacteria from a Ni rich serpentine environment and assessed their efficiency in promoting plant growth and Ni uptake in *Brassica* species. During the initial screening, 23 Ni (50 mg L^{-1}) resistant bacterial strains were isolated from the soil samples. In order to isolate the Ni mobilizing bacteria, the Ni resistant strains were tested for the ability to increase the water soluble Ni concentrations in soils. Among the 23 strains tested, nine strains namely SN3, SN9, SRS5, SRS8, SRS15, SRI2, SRI4, SRI11 and SRI14 significantly increased the concentrations of water soluble Ni in soil compared with non-inoculated control (Fig. 1). In general the soil bacteria have been known to exude biosurfactants, organic acids and to produce siderophores which stimulate metal bioavailability in soil and thereby facilitate their uptake through root absorption of various metal ions, including Fe^{2+} (Crowley et al., 1991), Mn^{2+} (Barber and Lee, 1974) and possibly Cd^{2+} (Salt et al., 1995). Enhanced Ni bioavailability by bioaugmentation of bacterial inocula (*Microbacterium arabinogalactanolyticum*) has been also reported by Abou-Shanab et al. (2003) from serpentine soils.

The Ni mobilizing strains exhibited a high tolerance to Ni when cultivated under increasing Ni levels in the growth medium (Table 1). Extremely high Ni resistance (up to the concentration of 1250 mg L^{-1}) was observed for the strains SRS5 followed by stains SN9, SRS8 and SRS15 (1000 mg L^{-1} Ni), whereas strain SN3 showed relatively low tolerance to Ni (500 mg L^{-1}). This high tolerance to Ni could be attributed to the fact that the bacteria were isolated from a serpentine soil containing high levels of Ni (Freitas et al., 2004). Microorganisms isolated from natural environments contaminated with heavy metals often exhibit tolerance to multiple

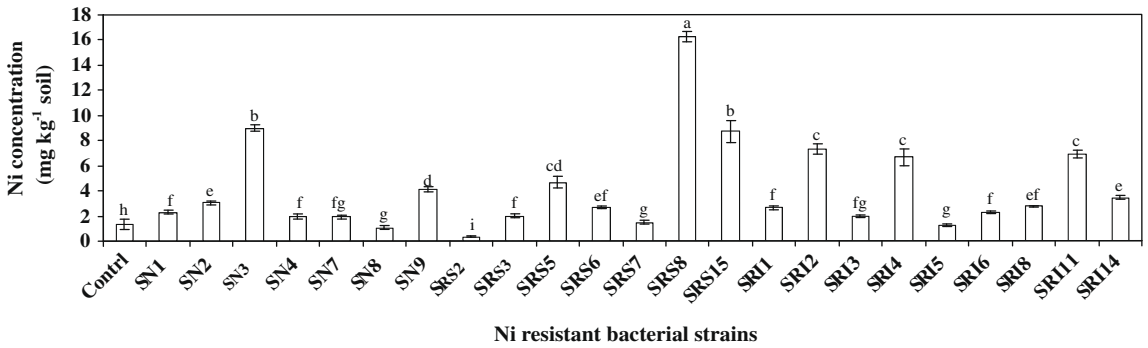

Fig. 1. Effects of inoculation with Ni resistant bacterial strains on the mobilization of Ni in soil. Each value is the mean of triplicates. Error bars represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

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Table 1
Bacterial strains used in this study.

Strain	Closest described relative	Accession no.	bp	Similarity (%)[a]	Origin	Ni tolerance level (mg L^{-1})
SN3	*Bacillus* sp.	FM205065	858	100	Non-rhizosphere serpentine soil	500
SN9	*Bacillus* sp.	FM205066	800	99	Non-rhizosphere serpentine soil	1000
SRS5	*Bacillus* sp.	FM205058	549	98	Rhizosphere of *Alyssum serpyllifolium*	1250
SRS8	*Psychrobacter* sp.	FM205059	810	96	Rhizosphere of *Alyssum serpyllifolium*	1000
SRS15	*Bacillus* sp.	FM205060	677	100	Rhizosphere of *Alyssum serpyllifolium*	1000
SRI2	*Pseudomonas* sp.	FM205061	511	87	Rhizosphere of *Astragalus incanus*	750
SRI4	*Bacillus* sp.	FM205062	786	99	Rhizosphere of *Astragalus incanus*	750
SRI11	*Bacillus* sp.	FM205063	685	99	Rhizosphere of *Astragalus incanus*	750
SRI14	*Bacillus* sp.	FM205064	858	94	Rhizosphere of *Astragalus incanus*	750

[a] Similarity is at the nucleotide level.

pollutants as they have adapted to such environments (Pal et al., 2005). Partial sequences of 16S rDNA of Ni mobilizing strains obtained were matched against nucleotide sequences present in GenBank using the basic local alignment search tool program. The strains SN3, SN9, SRS5, SRS15, SRI4, SRI11 and SRI14 were close to the members of the genus *Bacillus*. Strain SRS8 showed high similarity with *Psychrobacter* sp. as did SRI2 with *Pseudomonas* sp. The sequences were deposited at GenBank (Table 1). Further studies on the characterization of Ni mobilizing strains at the species level are under progress.

3.2. Plant growth-promoting features of Ni mobilizing bacteria

The importance of soil bacteria in heavy metal mobilization and their ability to promote the host plant growth in a metal-contaminated environment make them the preferred choice for the phytoremediation studies. In addition to metal mobilization, certain soil bacteria could exert their beneficial effects on host plant by several possible mechanisms. The mechanisms include: synthesis of ACC deaminase which can hydrolyze ACC (the immediate precursor of the plant hormone ethylene) (Honma and Shimomura, 1978; Adams and Yang, 1979); production of phytohormones, which can enhance the growth of plants (Xie et al., 1996); synthesis of siderophore, which can solubilize and sequester iron from the soil (Burd et al., 2000); and solubilization of phosphorus (Zaidi et al., 2006). Hence, the plant growth-promoting characteristics of Ni mobilizing strains were further investigated in detail.

The ACC deaminase activity of Ni mobilizing strains was determined by estimating the amount of α-KB generated by the enzymatic hydrolysis of ACC. Among the nine strains tested, SN9, SRI2, SRI4, SRI11 and SRI14 grew in Dworkin and Foster minimal salt medium (Dworkin and Foster, 1958) with ACC as the sole source of nitrogen (data not shown). However, strain SRI2 recorded the highest ACC deaminase activity followed by SRI4 (Table 2). The role of ACC deaminase in decreasing ethylene levels by the enzymatic hydrolysis of ACC into α-KB and ammonia has been presented as one of the major mechanisms of PGPB in promoting root and plant growth under metal stress condition (Glick et al., 1998). Further screening on the production of IAA by Ni mobilizing bacteria indicated that all strains utilized L-tryptophan as a precursor for growth and IAA production. However, strain SRS8 produced the highest amount, 110 mg L^{-1} of IAA, whereas SN3 produced only 14 mg L^{-1} of IAA (Table 2). Generally the PGPB have been reported to influence plant growth by contributing to the host plant's endogenous pool of phytohormones, such as IAA. A low level of IAA produced by PGPB promotes primary root elongation whereas a high level of IAA stimulates lateral and adventitious root formation but inhibit primary root growth (Xie et al., 1996). In addition to IAA production, the phosphate solubilization by PGPB is believed to play an important role in plant–bacterial interactions and plant growth in metal-contaminated soils (Zaidi et al., 2006). In the present study, all nine strains showed the phosphate solubilizing ability by utilizing the insoluble tricalcium phosphate in modified Pikovskayas medium. However, maximum solubilization of phosphate was recorded in the strains SRS8 (126 mg L^{-1}) and SRI14 (124 mg L^{-1}) (Table 2).

Siderophores are another important metabolite released by the plant growth-promoting rhizobacteria that indirectly alleviate heavy metal toxicity by increasing the supply of iron to the plant (Burd et al., 2000). In the present study, production of siderophores by serpentine isolates was determined using CAS assay (Schwyn and Neilands, 1987). All the strains, except strain SRS5 displayed a positive siderophore activity, as indicated by the development of orange-colored zone on CAS agar plates, after 5 d of growth. Furthermore, the production of catechol and hydroxamate siderophores was also determined. The maximum production of catechol siderophore was recorded in the strains SN9, SRS8 and SRI2 (Table 2). Similarly, the strain SN9 recorded maximum production of hydroxamate siderophores followed by SRI2. Crowley et al. (1988) previously reported the existence of a siderophore-

Table 2
Plant growth-promoting features of Ni mobilizing bacterial strains.

Strain	ACC deaminase (μm α-KB mg^{-1} h^{-1})	Phosphate solubilization (mg L^{-1})	IAA synthesis (mg L^{-1})	Siderophore production		
				CAS assay (cm)	Catechol (mg L^{-1})	Hydroxamate (mg L^{-1})
SN3	nd	114 ± 4^{c}	14 ± 1^{e}	1.9 ± 0.1^{b}	53 ± 14^{h}	19 ± 1^{e}
SN9	19 ± 2^{c}	101 ± 3^{d}	72 ± 1^{d}	2.3 ± 0.1^{a}	1081 ± 47^{a}	92 ± 3^{a}
SRS5	nd	109 ± 2^{c}	21 ± 0^{g}	nd	nd	nd
SRS8	nd	126 ± 2^{a}	111 ± 1^{a}	2.0 ± 0.1^{ab}	899 ± 30^{b}	28 ± 2^{d}
SRS15	nd	111 ± 1^{c}	84 ± 3^{b}	1.9 ± 0.1^{b}	103 ± 15^{g}	22 ± 1^{e}
SRI2	73 ± 3^{a}	119 ± 3^{b}	61 ± 1^{f}	2.1 ± 0.1^{ab}	615 ± 28^{c}	59 ± 2^{b}
SRI4	26 ± 1^{b}	119 ± 4^{b}	68 ± 1^{e}	2.2 ± 0.2^{a}	435 ± 21^{e}	50 ± 8^{c}
SRI11	16 ± 1^{c}	123 ± 2^{ab}	77 ± 3^{c}	2.1 ± 0.1^{ab}	528 ± 16^{d}	53 ± 1^{c}
SRI14	11 ± 2^{d}	125 ± 2^{a}	20 ± 1^{g}	2.1 ± 0.3^{ab}	228 ± 24^{f}	31 ± 2^{d}

Average ± standard deviation from three samples. Nd: not detected. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

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mediated iron-transport system in oats and suggested that siderophores produced by rhizosphere microorganisms can supply iron to plants that have mechanisms for using these compounds under iron-limited conditions.

The production of ACC deaminase, IAA, siderophores and solubilization of phosphate by Ni mobilizing bacteria indicate their inherent plant growth-promoting potential. Considering such potential, the efficiency of Ni mobilizing PGPB on plant growth and Ni uptake by *Brassica* species was tested in Ni contaminated soils.

3.3. Influence of Ni mobilizing PGPB on plant growth and Ni uptake

Nickel in excessive concentration disturbs photosynthetic electron transport (Mohanty et al., 1989) as well as inhibits CO_2 assimilation and stomatal conductance (Sheoran et al., 1990). In our experiment, the non-inoculated plants exposed to 450 mg Ni kg^{-1} demonstrated a significant ($p < 0.05$) inhibition in plant growth. It has earlier been reported that increasing Ni supply resulted in decreased plant biomass indicating the alterations of physiology and metabolism of test plants (Zaidi et al., 2006). *B. juncea* inoculated with Ni mobilizing strains exhibited an increase in plant fresh and dry weight in the presence of Ni. Among nine strains, the three best performers with respect to plant growth promotion potential were recorded as SRI2 > SRS8 > SN9. Similarly, *B. oxyrrhina* inoculated with Ni mobilizing strains exhibited an increase in plant growth. On the basis of *B. oxyrrhina* growth promotion, the bacterial strains may be arranged in the order as: SRI2 > SN9 > SRS8. The results obtained here clearly indicate that inoculation with Ni mobilizing PGPB especially SRI2, SRS8 and SN9 was highly efficient at protecting the plants from growth inhibition caused by toxic soil Ni concentrations.

The PGPB inoculation has been reported to have a positive influence on various plant growth parameters, including root and shoot length, fresh and dry biomass (Zaidi et al., 2006). ACC deaminase producing bacteria have been reported to prevent the inhibition of root elongation by decreasing the level of growth-limiting ethylene through hydrolytic cleavage (deaminase activity) of the ethylene biosynthesis precursor ACC (Adams and Yang, 1979; Glick et al., 1998). However, in our study the significant increase in the fresh and dry biomass of the crop plants treated with ACC deaminase-negative strain SRS8 (Table 3) suggests that the effects of other plant growth-promoting features such as the production of IAA, siderophore and solubilization of phosphate should also be considered in addition to ACC deaminase activity. In general, the reduction of plant growth in metal-contaminated soil is often associated with iron deficiency and reduced uptake of phosphate (Halstead et al., 1969; Burd et al., 2000). Inoculation of phosphate solubilizing and siderophore producing PGPB might have helped plant root proliferation and enhanced the uptake of soil minerals such as P and Fe by the host plant (Braud et al., 2006; Zaidi et al., 2006). Further, IAA produced by PGPB promotes the root growth by directly stimulating plant cell elongation or cell division (Glick et al., 1998). In addition, earlier studies have reported that the plant growth under adverse environmental condition is correlated with population density of beneficial bacteria in the rhizosphere or roots (Ashraf et al., 2004). Hence, in this study, the population densities of Ni mobilizing PGPB in the rhizosphere of *B. juncea* and *B. oxyrrhina* were analyzed. In general, the Ni mobilizing PGPB strains colonized comparatively better on the *B. juncea* rhizosphere soil than on the *B. oxyrrhina* rhizosphere soil (Fig. 2). The data indicate that colonization efficiency of PGPB may depend on specific interactions between bacterial species and host plants. Though the strains SN9 and SRS8 were isolated from the non-rhizosphere soil and the rhizosphere soil of *A. serpyllifolium*, respectively, they showed high level of colonization on the rhizosphere of both *B. juncea* and *B. oxyrrhina*. The observations indicate that there was a significant relationship between the plant growth and the population density of added PGPB on the rhizosphere. In general, the potential of root colonization by inoculated PGPB depends on various parameters such as bacterial traits, root exudates, biotic and abiotic factors (Benizri et al., 2001). Hence, the interactions between root exudates and PGPB, and their influence on biochemical changes in the rhizosphere need to be investigated further to know the factors involved in root colonization by both the strains of this study. The findings, therefore, suggest successful colonization and subsequent plant growth-promoting potentiality of SN9 and SRS8 in both of the plants (Fig. 2).

The accumulation and distribution of metals in the plant tissue are important aspects to evaluate the role of plants in remediation of contaminated sites (Kumar et al., 1995). In the present study, we assessed whether inoculation with Ni mobilizing PGPB strains affected the uptake of Ni by *B. juncea* and *B. oxyrrhina* plants (Fig. 3). In general, *B. oxyrrhina* accumulated more Ni in both the shoot and root tissues compared with *B. juncea*. Further, the inoculation of Ni mobilizing strains significantly increased the accumu-

Table 3
Influence of Ni mobilizing bacteria on the fresh weight and dry weight of *Brassica* species.

Bacterial strain	*B. juncea*		*B. oxyrrhina*	
	Fresh weight (mg $plant^{-1}$)	Dry weight (mg $plant^{-1}$)	Fresh weight (mg $plant^{-1}$)	Dry weight (mg $plant^{-1}$)
Control	291 ± 1^{d}	16.5 ± 0.2^{c}	189 ± 6^{b}	8.5 ± 0.4^{b}
Ni	260 ± 1^{e}	14.9 ± 0.3^{d}	153 ± 1^{e}	8.0 ± 0.1^{c}
SN3	270 ± 2^{e}	15.1 ± 0.2^{d}	164 ± 2^{d}	8.4 ± 0.2^{b}
SN9	312 ± 1^{b}	17.1 ± 0.2^{a}	197 ± 1^{a}	9.5 ± 0.3^{a}
SRS1	284 ± 2^{d}	$16.1 \pm 0.3^{b,c}$	176 ± 1^{c}	8.4 ± 0.8^{b}
SRS5	265 ± 4^{e}	15.5 ± 0.2^{c}	160 ± 5^{d}	8.5 ± 0.2^{b}
SRS8	321 ± 2^{a}	17.2 ± 0.4^{a}	194 ± 2^{a}	9.4 ± 0.1^{a}
SRS15	268 ± 5^{e}	15.9 ± 0.6^{c}	162 ± 3^{d}	8.6 ± 0.2^{b}
SRI2	322 ± 6^{a}	17.3 ± 0.4^{a}	199 ± 1^{a}	9.6 ± 0.2^{a}
SRI4	295 ± 4^{c}	17.2 ± 0.4^{a}	191 ± 1^{b}	9.3 ± 0.1^{a}
SRI11	294 ± 3^{c}	17.0 ± 0.7^{a}	189 ± 3^{b}	9.2 ± 0.1^{a}
SRI14	285 ± 4^{d}	16.6 ± 0.5^{b}	176 ± 8^{c}	8.7 ± 0.2^{b}

Average ± standard deviation from three samples. nd: not detected. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

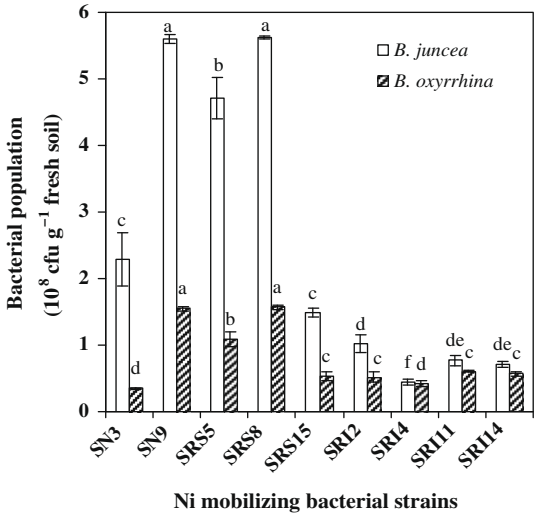

Fig. 2. Colonization potential of Ni mobilizing bacterial strains in rhizosphere soil of *B. juncea* and *B. oxyrrhina*. Each value is the mean of triplicates. Error bars represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

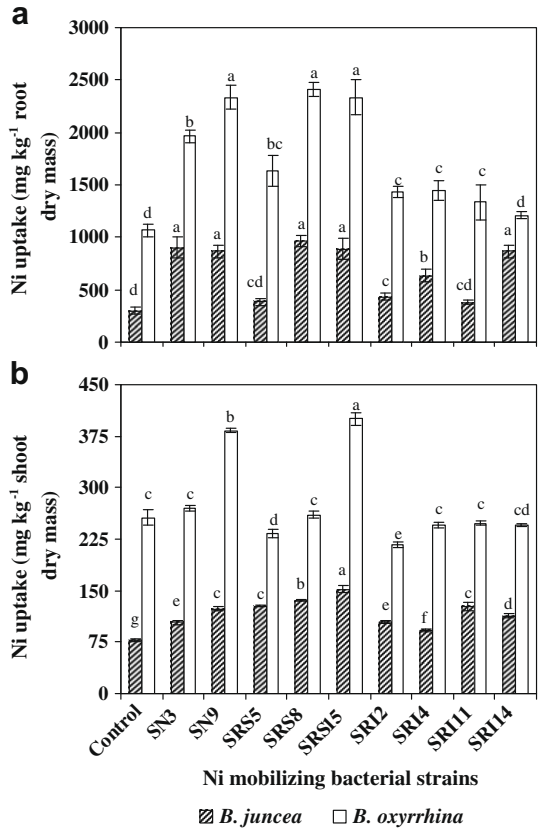

Fig. 3. Ni concentration (mg kg^{-1}) in root (a) and shoot system (b) of *B. juncea* and *B. oxyrrhina*. Each value is the mean of triplicates. Error bars represent standard deviation. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

lation of Ni in the root tissues of both *B. juncea* and *B. oxyrrhina* compared with respective non-inoculated controls (Fig. 3a). For instance, strain SRS8 increased the Ni concentration in the root tissues of *B. juncea* and *B. oxyrrhina* by 222% and 126%, respectively. In contrast to the present observation, Vivas et al. (2006) reported that inoculation with PGPB *Brevibacillus* sp. reduced Zn uptake in *Trifolium repens* plants. Previously, *B. juncea* grown in nickel amended soil were found to be able to accumulate significant amount of nickel in their shoots (Kumar et al., 1995). However, in the work reported here, it was found that the inoculated and non-inoculated root tissues accumulated considerably more Ni as compared to the respective shoot tissues. This can be attributed to poor translocation of nickel from root to shoot system (Burd et al., 2000). Further, the inoculation of bacterial strains did not greatly influence the accumulation of Ni in shoot tissues as compared to the root tissues of respective plants. However, the strains SN9 and SRS15 significantly increased the accumulation of Ni in the shoot tissues of both *B. juncea* and *B. oxyrrhina* compared with respective non-inoculated controls (Fig. 3b). For instance, strain SN9 increased the Ni concentration in the shoot tissues of *B. juncea* and *B. oxyrrhina* by 58% and 49%, respectively. Studies have evidenced that heavy metal resistant bacteria can enhance metal uptake by hyperaccumulator plants (Whiting et al., 2001) as has shown in our experiment that the Ni uptake by *B. juncea* and *B. oxyrrhina* was enhanced by the Ni resistant PGPB SN9 and SRS8.

In order to confirm the correlation between bacterial Ni mobilization in soil and total Ni accumulation in plants, we performed a regression analysis of bacterial Ni mobilization and the Ni accumulation in plants that had been inoculated with the corresponding isolate. A positive correlation was observed between the bacterial Ni mobilization in soil and the total Ni uptake in both *B. juncea* ($R^2 = 0.390$, $p = 0.022$) and *B. oxyrrhina* ($R^2 = 0.471$, $p = 0.007$) (Fig. 4). The Ni mobilizing PGPB, SRS8 and SRS15 had maximum Ni mobilization potential and they were also found to be most effective in promoting Ni accumulation in both *B. juncea* and *B. oxyrrhina*. Our result indicates that Ni mobilizing PGPB facilitated the release of Ni from the non-soluble phases in the soil, thus enhancing the availability of Ni to plants. These effects of inoculation were reported also by Rajkumar and Freitas (2008), who found that the addition of *Pseudomonas jessenii* to surface sterilized root of *Ricinus communis* in autoclaved soil increased Ni, Cu and Zn concentrations in root tissues compared with non-inoculated controls. Possible explanations include soil acidification, release of siderophores and phosphate solubilization (Abou-Shanab et al., 2005; Zaidi et al., 2006). Although the microbial activity strongly influences metal speciation and transport in environment, further studies including e.g. detailed chemical (metabolite) and microbial analysis in the rhizosphere are required to better understand the interactions between microorganisms and heavy metal accumulating plants and to elucidate the mechanisms how bacteria can promote heavy metal accumulation and translocation in plants.

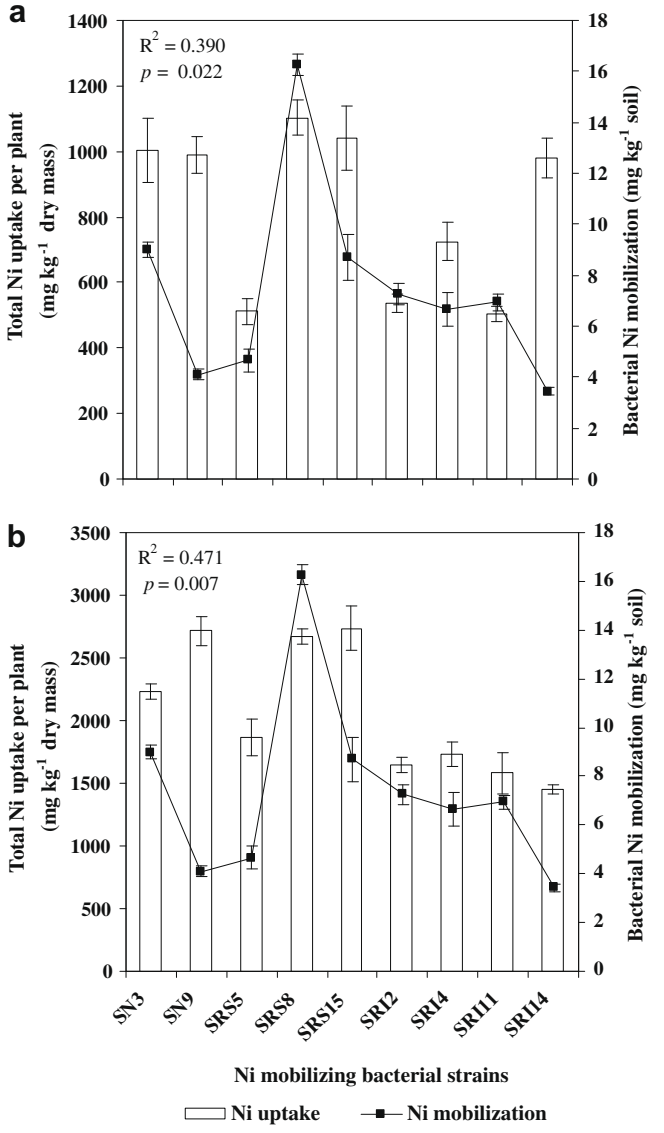

Fig. 4. (a) Regression of bacterial Ni mobilization in soil and the total Ni uptake in *B. juncea* inoculated with bacterial isolates. (b) Regression of bacterial Ni mobilization in soil and the total Ni uptake in *B. oxyrrhina* inoculated with bacterial isolates.

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4. Conclusions

The results obtained here indicate that inoculation of Ni mobilizing PGPB, *Pseudomonas* sp. SRI2, *Psychrobacter* sp. SRS8 and *Bacillus* sp. SN9 seemed to be very effective in protecting plants from growth inhibition caused by Ni. As successful bioinoculant for microbial-assisted phytoremediation, the microorganisms must be able to promote plant growth and heavy metal uptake. In the present study, though the strains SRI2 and SRS8 significantly increased the growth of *B. juncea* and *B. oxyrrhina* in Ni contaminated soils, they did not greatly influence the quantity of accumulation of Ni in shoot tissues of *B. oxyrrhina* plants. However, the strain SN9 not only significantly increased the Ni concentration in the root and shoot tissues of both *B. juncea* and *B. oxyrrhina*, but also promoted the plant growth against the toxic effects of Ni in soils. The increase in plant growth caused by strain SN9 may be attributed to the production of IAA, siderophore and solubilization of phosphate. The findings, therefore, revealed that inoculation of Ni mobilizing PGPB strain SN9 increases the efficiency of phytoextraction directly by enhancing Ni accumulation in plant tissues and indirectly by promoting the shoot and root biomass of *B. juncea* and *B. oxyrrhina.* Due to multifarious properties expressed by the Ni mobilizing PGPB in this study, the strain SN9 could therefore, be used as bioinoculant to increase the phytoremediation potential of hyperaccumulators in soils contaminated with nickel. However, it should also be mentioned that these experiments were performed under laboratory conditions and in a sterile environment without any interference of competing microorganisms that are present in soil. Further works including the population dynamics and activity of inoculated bacteria in their host plants, the mechanisms involved in Ni mobilization by bacteria, and the basic mechanisms of plant–microbe interactions in the rhizosphere are required to implement these microbial-assisted phytoremediation in field level.

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